Reference: *Biol. Bull.* **199:** 305–315. (December 2000)

Development of Embryonic Cells Containing Serotonin, Catecholamines, and FMRFamide-Related Peptides in *Aplysia californica*

AMANDA J. G. DICKINSON[1,*], ROGER P. CROLL[1], AND ELENA E. VORONEZHSKAYA[2]

[1] *Department of Physiology and Biophysics, Faculty of Medicine, Dalhousie University, Halifax, Nova Scotia, B3H 4H7, Canada; and* [2] *Institute of Developmental Biology, Russian Academy of Sciences, 117808 Moscow, Russia*

Abstract. This study demonstrates the presence of a relatively extensive but previously unrecognized nervous system in embryonic stages of the opisthobranch mollusc *Aplysia californica*. During the trochophore stage, two pairs of cells were observed to be reactive to antibodies raised against the neuropeptides FMRFamide and EFLRIamide. These cells were located in the posterior region of the embryo, and their anterior projections terminated under the apical tuft. As the embryos developed into veliger stages, serotonin-like immunoreactive (LIR) cells appeared in the apical organ and were later observed to innervate the velum. Also, aldehyde-induced fluorescence indicative of catecholamines was present in cells in the foot, oral, and possibly apical regions during late embryonic veliger stages. Just before the embryo hatches as a free-swimming veliger, additional FMRFamide-LIR and catecholamine-containing cells appeared in regions that correspond to the ganglia of what will become the adult central nervous system (CNS). Neurons and connectives that will contribute to the adult CNS appear to develop along the pathways that are pioneered by the earliest posterior FMRFamide-LIR cells. These observations are consistent with the hypothesis that, besides their presumed roles in the control of embryonic behaviors, some elements may also guide the development of the CNS. Embryonic nervous systems that develop prior to and outside of the adult CNS have also been reported in pulmonate and prosobranch species of molluscs. Therefore, the demonstration of early developing neurons and their transmitter phenotypes in *A. californica* presents new opportunities for a better understanding of the ontogeny and phylogeny of both behavioral and neuronal function in this important model species.

Received 24 January 2000; accepted 2 October 2000.

* To whom correspondance should be addressed. E-mail: ajdickin@is2.dal.ca

Abbreviations: ACP, 35 amino acid acidic peptide; CC-1, catecholaminergic central cell one; CC-2, catecholaminergic central cell two; CC-3, catecholaminergic central cell three; CNS, central nervous system; EFLRIamide, Glu-Phe-Leu-Arg-Ile-NH_2; EDTA, ethylenediaminetetraacetic acid; FC-1, FMRFamide central cell one; FC-2, FMRFamide central cell two; FC-3, FMRFamide central cell three; FITC, fluorescein isothiocyanate; F-*l*1, left FMRFamide-LIR posterior cell one; F-*l*2, left FMRFamide-LIR posterior cell two; F-*r*1, right FMRFamide-LIR posterior cell one; F-*r*2, right FMRFamide-LIR posterior cell two; FMRFamide, Phe-Met-Arg-Phe-NH_2; LIR, like-immunoreactive; PBS, phosphate-buffered saline; SEEPLY, 22 amino acid peptide SEQPDVDDYLRDVVLQSEEPLY; S-*l*1, left serotonin-LIR bilateral cell one; S-*l*2, left serotonin-LIR bilateral cell two, S-*r*1, right serotonin-LIR bilateral cell one; S-*r*2, right serotonin-LIR bilateral cell two; SUM, serotonin-LIR unpaired median cell.

Introduction

The opisthobranch gastropod *Aplysia californica*, which has been studied extensively as a model for understanding the neuronal underpinnings of behavior (for review see Kandel [1979]), has also become an important model in the study of molluscan neurodevelopment. The development of the ganglia that constitute the central nervous system (CNS) has been studied in detail (Kriegstein, 1977; Schacher *et al.*, 1979; Kandel *et al.*, 1981; Jacob, 1984), but more recent studies also report the presence of nerve cells that exist outside the boundaries of the developing ganglia that will constitute the adult CNS. For example, in an early embryonic veliger stage, three serotonin-like immunoreactive (LIR) cells exist in the anterior apical organ: an unpaired median cell and a bilateral pair of cells (Croll and Voron-

ezhskaya, 1995; Croll and Voronezhskaya, 1996b; Marois and Carew, 1997a, b, c). Soon afterwards, these three cells are joined by another more lateral pair of apical serotonin-LIR cells. By the end of the embryonic period, as the veliger is about to hatch as a free-swimming larva, serotonin-LIR projections extend into the velar lobes, foot, and abdominal and visceral regions (Marois and Carew, 1997a, b, c). Kempf *et al.* (1997) showed that such apical cells and processes appear to be general features of opisthobranch larvae. Similarly shaped and positioned apical cells have also been reported in larvae of other molluscan species (Bonar, 1978; Kulakovskiy and Flyachinskaya, 1994; Raineri and Ospovat, 1994; Raineri, 1995; Leise, 1996; Lin and Leise, 1996a, b; Dickinson *et al.*, 1999; Voronezhskaya *et al.*, 1999; Parries, 2000).

In addition to the cells of an apical organ, other neuronal elements have been observed outside the boundaries of the developing adult CNS. Croll and Voronezhskaya (1996b) reported preliminary observations of elements containing peptides related to Phe-Met-Arg-Phe-NH_2 (FMRFamide) in posterior regions of embryonic *A. californica*. Recent studies also indicate the presence of similar neuronal elements in other molluscan species. For example, Croll and Voronezhskaya (1995; 1996a) identified neuronal elements in what corresponds to the trochophore or early veliger stage (Mescheryakov, 1990) of the pulmonate *Lymnaea stagnalis*, using antibodies raised against FMRFamide. These FMRFamide-LIR cells develop in posterior regions of the embryo and send anterior projections that terminate in the regions of the future cerebral and pedal ganglia. A posterior FMRFamide-LIR cell has also been observed in the early developmental stages of the prosobranch *Crepidula fornicata* (Dickinson *et al.*, 1999). As in *L. stagnalis*, this posterior FMRFamide-LIR cell also sends anterior projections that terminate in the region of the future cerebral and pedal ganglia. Therefore, cells expressing FMRFamide-like immunoreactivity appear to develop in a posterior-to-anterior sequence rather than the anterior-to-posterior development of the ganglia. In addition, these FMRFamide-LIR cells and their fibers seem to mark the pathways along which the adult ganglia and connectives develop, and therefore they may be involved in guiding the developing CNS.

Additional peripherally located neurons in the foot and surrounding the mouth were revealed in the gastropods *L. stagnalis* (Voronezhskaya *et al.*, 1999), *C. fornicata* (Dickinson *et al.*, 1999), and *Phestilla sibogae* (Pires *et al.*, 2000) and the bivalve *Mytilus edulis* (Croll *et al.*, 1997), using techniques to localize catecholamines.

The above descriptions of neurodevelopment in representative species suggest the presence of a primary larval nervous system that appears earlier than and outside of the developing adult CNS. Morphological descriptions in other species also indicate that components of such primary larval nervous systems may either be incorporated into the adult ganglia or disappear. The present study investigated the early development of neurons that may compose a primary larval nervous system in *A. californica*, starting at the trochophore stage and continuing until the embryo hatches as a free-swimming veliger. We used immunocytochemical techniques to study the first cells expressing FMRFamide and related peptides, and we provide details of the morphology of these cells and the timing of their appearance, with comparisons to the earliest cells exhibiting serotonin-like immunoreactivity. We additionally used aldehyde-induced fluorescence, which has been previously applied to molluscan tissues (Croll *et al.*, 1997, 1999; Smith *et al.*, 1998), to examine cells containing catecholamines. This study shows that, as in these other molluscs, *A. californica* also has a nervous system first present in early embryonic stages. Furthermore, these observations suggest several new hypotheses regarding the mechanism shaping the ontogeny of the nervous system in this well-studied species.

Materials and Methods

Animals

Adult specimens of *Aplysia californica* were purchased from the Aplysia Resource Facility of the University of Miami and maintained in a salt-water aquarium. Egg masses were collected soon after oviposition and kept in separate containers of artificial salt water (Crystal Sea, Baltimore, MD) at 20°–22°C. Under these conditions, embryos required about 9–10 days to develop from first cleavage to hatching. The hatched veliger required another few weeks before becoming competent to metamorphose into juvenile sea slugs (Kandel, 1979; Kandel *et al.*, 1981; Marois and Croll, 1992; Marois and Carew, 1997b).

The developmental stages of *A. californica* were described previously (Kriegstein, 1977; Kandel, 1979; Kandel *et al.*, 1981; Marois and Croll, 1992; Marois and Carew, 1997b). In the present study, morphological and behavioral features were examined on each day from first cleavage to hatching, and the embryonic development was divided into three stages. During the trochophore stage (days 2.5–4) the embryo had a distinct apical tuft and a shell gland and began to move using the prototrochal cilia. On day 4 the body began to change shape as the rudiments of a velum, foot, and shell were observed. During the early embryonic veliger stage (days 5–7) the velum became bilobate and possessed long cilia along its edge. During the late embryonic veliger stage (days 8–10) the velum, foot, and shell enlarged and differentiated.

Immunocytochemistry

Immunohistological procedures were performed according to Marois and Croll (1992) and Marois and Carew (1997b). Egg ribbons were fixed in 4% paraformaldehyde in

phosphate-buffered saline (PBS; 50 m*M* $Na_2HPO_4 \cdot 7H_2O$ and 140 m*M* NaCl in distilled water adjusted to pH 7.2) for 1–4 h at room temperature. Then embryos were removed from the capsules, washed in PBS, and stored in 70% ethanol at −18°C until further processing. For immunohistochemical processing, the stored embryos were first given two to three 5-min washes in PBS. The shells of older embryos (>day 4) were then decalcified with 10% ethylenediaminetetraacetic acid (EDTA) (Sigma Chemical Co., Mississauga, ON) in PBS for 30–45 min. Embryos were next washed for 2–3 h in 4% Triton X-100 in PBS. The embryos were then incubated in antibodies raised against FMRFamide, serotonin (both obtained from Diasorin, Stillwater, MI), or antibodies (gifts from Dr. P. R. Benjamin, University of Sussex) against three FMRFamide gene encoding peptides: the pentapeptide Glu-Phe-Leu-Arg-Ile-NH_2 (EFLRIamide), the 22-amino-acid peptide SEQPDVDDYLRDVVLQSEEPLY (SEEPLY), and a 35-amino-acid acidic peptide, SDPFFRFGKQQVATDDSGELDDEILSRVSDDDKNI (ACP) (Santama *et al.*, 1996). All these antibodies except anti-SEEPLY were diluted 1:500–1:1000 in PBS with the addition of 1.0% normal goat serum and 1.0% Triton X-100. The SEEPLY antibody was diluted 1:200 in a solution of 50 m*M* Tris base, 150 m*M* NaCl, pH 7.6, containing 0.25% w/v gelatin and 1% v/v Trition X-100 (Santama *et al.*, 1993). Incubation periods lasting 48 h at 4°C or 12 h at room temperature gave comparable results. The embryos were next rinsed three times (5 min each) with PBS and given a final wash for 1 h before incubating for 24–48 h in goat anti-rabbit antibodies conjugated to fluorescein isothiocyanate (FITC) or rhodamine (Bio/Can Scientific, Mississauga, Ontario) and diluted 1:50 in PBS with the addition of 1.0% Triton X-100.

To localize FMRFamide-like immunoreactivity relative to external morphological structures at the trochophore stage, some embryos were double-labeled with monoclonal antibodies against ∝-tubulin (DM1A clone from Sigma Chemical Co., Mississauga, ON) (Jackson *et al.*, 1995). These embryos were first labeled, as described above, for FMRFamide-like immunoreactivity, then rinsed three times (5 min each) in PBS. Next, the embryos were incubated in anti-∝tubulin (diluted 1:500 in PBS) for 12 h at room temperature. The embryos were washed again three times in PBS before incubating in sheep anti-mouse serum conjugated to FITC or rhodamine for 12 h at room temperature. These secondary antibodies were diluted 1:50 in PBS and 1% Triton X-100.

Embryos processed for immunocytochemistry were mounted on glass slides in a 3:1 mixture of glycerol to PBS for viewing on a Leitz Aristoplan microscope equipped for epifluorescence. FITC fluorescence was viewed using a 450–490-nm excitation filter and a 525/20-nm barrier filter; rhodamine fluorescence was viewed using a 530–560-nm excitation filter and 580-nm long-pass barrier filter. Embryos processed for FMRFamide- and ∝-tubulin-like immunoreactivity were also viewed on a Zeiss Axiovert microscope equipped for confocal laser scanning (model LSM 410).

As negative controls, embryos were processed without incubation in primary antibody; such specimens exhibited no detectable fluorescence. Positive controls involved parallel processing of embryonic *L. stagnalis* that exhibited typical staining, as described elsewhere for serotonin and FMRFamide (Marois and Croll, 1992; Croll and Voronezhskaya, 1995; Croll and Voronezhskaya, 1996a).

Catecholamine histofluorescence

The formaldehyde glutaraldehyde technique of Furness *et al.* (1977) was used to localize catecholamines. Embryos were incubated for at least 12 h in a solution consisting of 4% paraformaldehyde, 0.5% glutaraldehyde, and 35% sucrose in PBS. Similar results were also obtained when the embryos were stored in this solution for several weeks. The fixed embryos were decalcified in 10% EDTA in PBS for 45 min. Embryos were then placed on glass slides, air dried for several hours, and then mounted in a 3:1 mixture of glycerol and PBS. These embryos were viewed and photographed through the Leitz compound microscope equipped with a 355–425-nm excitation filter and 460-nm long-pass barrier filter. Positive controls involved parallel processing of embryonic *L. stagnalis* that exhibited typical blue-green fluorescent staining, as described elsewhere for catecholamines (Voronezhskaya *et al.*, 1999). Negative controls were performed by omitting the glutaraldehyde from the formaldehyde glutaraldehyde solution, thus eliminating the characteristic fluorescent staining.

Photography

Most histological preparations were photographed on the Leitz compound microscope using Kodak TMAX 100 film; the negatives were digitally scanned. Photographs from the Zeiss confocal microscope were produced by superpositioning stacks of 10–15 images obtained through stepped sequences of focal planes at intervals of 1–2 μm. All the images were then assembled into plates and labeled using Photoshop 5.0 (Adobe Systems, Inc., San Jose, CA). Contrast and brightness of the images were adjusted to provide consistency within plates.

Results

Trochophore stage (days 2.5–4)

Halfway through day 2, two bilaterally symmetrical pairs of FMRFamide-LIR posterior cells were observed (Figs. 1A, 2A). Fibers projected ipsilaterally and anteriorly from each cell on the right (F-*r*1 and F-*r*2) and the left (F-*l*1 and F-*l*2) (Fig. 1A). By day 3 these fibers terminated in a plexus

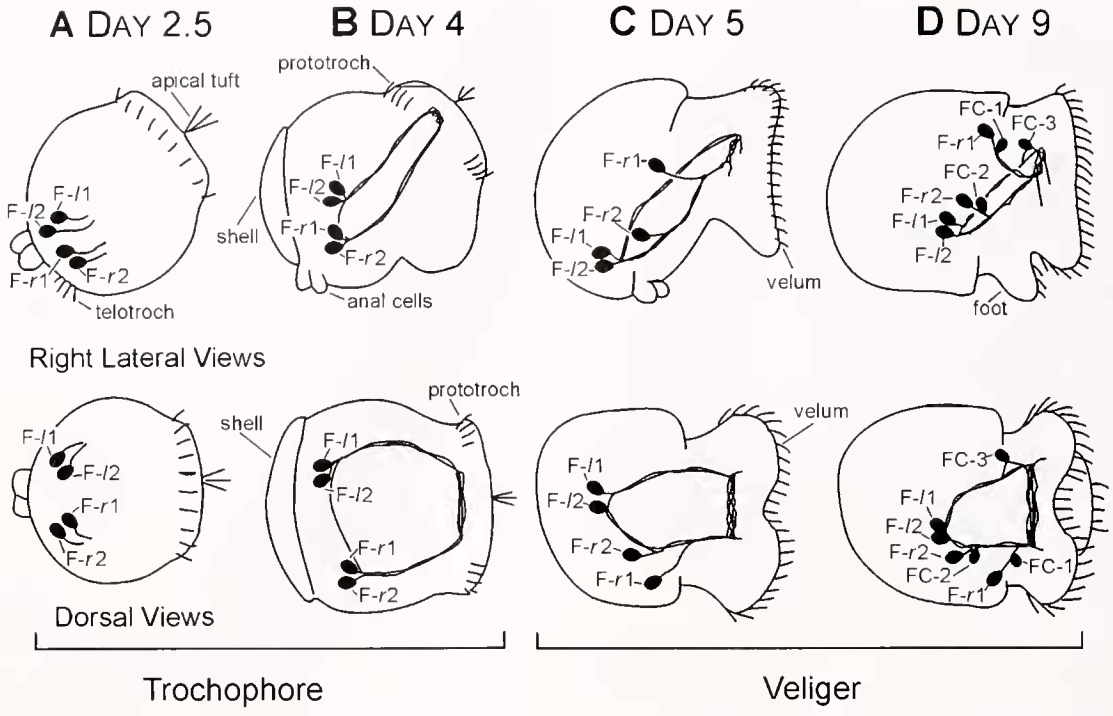

Figure 1. Schematic representations of FMRFamide-LIR cells and fibers in embryos of *Aplysia californica.* Top row: views from the right side and slightly superior to give a three-dimensional perspective; bottom row: dorsal views. Anterior is to the right in each figure. (A) Two pairs of posteriorly located FMRFamide-LIR cells with anterior projections observed on day 2.5. (B) FMRFamide-LIR cells and their processes observed on day 4. The FMRFamide-LIR processes reached the anterior region, where they formed a plexus under the apical tuft. (C) FMRFamide-LIR cells were no longer symmetrical by day 5; the cells (*l*1 and *l*2) on the left appeared in a ventral position, and the cells on the right (*r*1 and *r*2) appeared more dorsally. (D) On day 9 additional FMRFamide-LIR cells (FC-1, FC-2, FC-3) appeared in the anterior region.

of FMRFamide-LIR processes in the region beneath the apical tuft (see day 4, Figs. 1B, 2B, D). Also by day 3, one to two additional FMRFamide-LIR fibers extended across the midline of the body just anterior to the somata of the FMRFamide-LIR posterior cells (See day 4, Figs. 1B, 2D). Initially the pairs of FMRFamide-LIR cells were positioned symmetrically within the embryo, but they gradually became displaced and by the end of day 4 had all moved to the right side of the body (Fig. 1C). The FMRFamide-LIR cells and processes were also identified using antibodies against EFLRIamide (Fig. 2C). No immunoreactivity was detected during the trochophore stage or any later stages with antibodies against SEEPLY and ACP.

Early embryonic veliger stage (days 5–7)

By day 5 the FMRFamide-LIR cells had assumed more anterior positions in the embryo. F-*r*1 and F-*r*2 moved apart from each other, with F-*r*1 occupying a more dorsal location. F-*l*1 and F-*l*2 remained close to each other and together assumed a central and ventral position (Figs. 1C, 3A). Their anteriorly projecting fibers crossed the midline and formed a commissure in the anterior region. All cells and processes listed above were also identified using antibodies against EFLRIamide (Fig. 3B).

Also by day 5, a serotonin-LIR unpaired median cell (SUM; see Marois and Carew [1997b]) appeared beneath the apical tuft. Soon afterwards, a pair of vase-shaped serotonin-LIR cells (S-*r*1 and S-*l*1) were observed to the left and right of SUM. Short serotonin-LIR fibers projected ventrally from the SUM, S-*r*1, and S-*l*1 to form a plexus in the same region as the FMRFamide-LIR commissure (see day 9, Figs. 4A, 5A, B, also see Marois and Carew [1997b]).

By day 7 the three serotonin-LIR cells (SUM, S-*r*1, and S-*l*1) were joined by a new pair of serotonin-LIR cells (S-*r*2 and S-*l*2) located slightly posteroventrally to S-*r*1 and S-*l*1 on either side of the plexus (see day 9, Figs. 4A, 5A, B).

Late embryonic veliger on prehatching stage (days 8–10)

By day 8 the posterior FMRFamide-LIR cells and their fibers appeared just anterior to the midpoint along the anteroposterior axis. F-*l*1 and F-*l*2 together assumed a ventral and central position, while F-*r*1 and F-*r*2 each assumed more dorsal positions than previously (see day 9, Figs. 1D, 3C). FMRFamide-LIR fibers extended ventrally from the

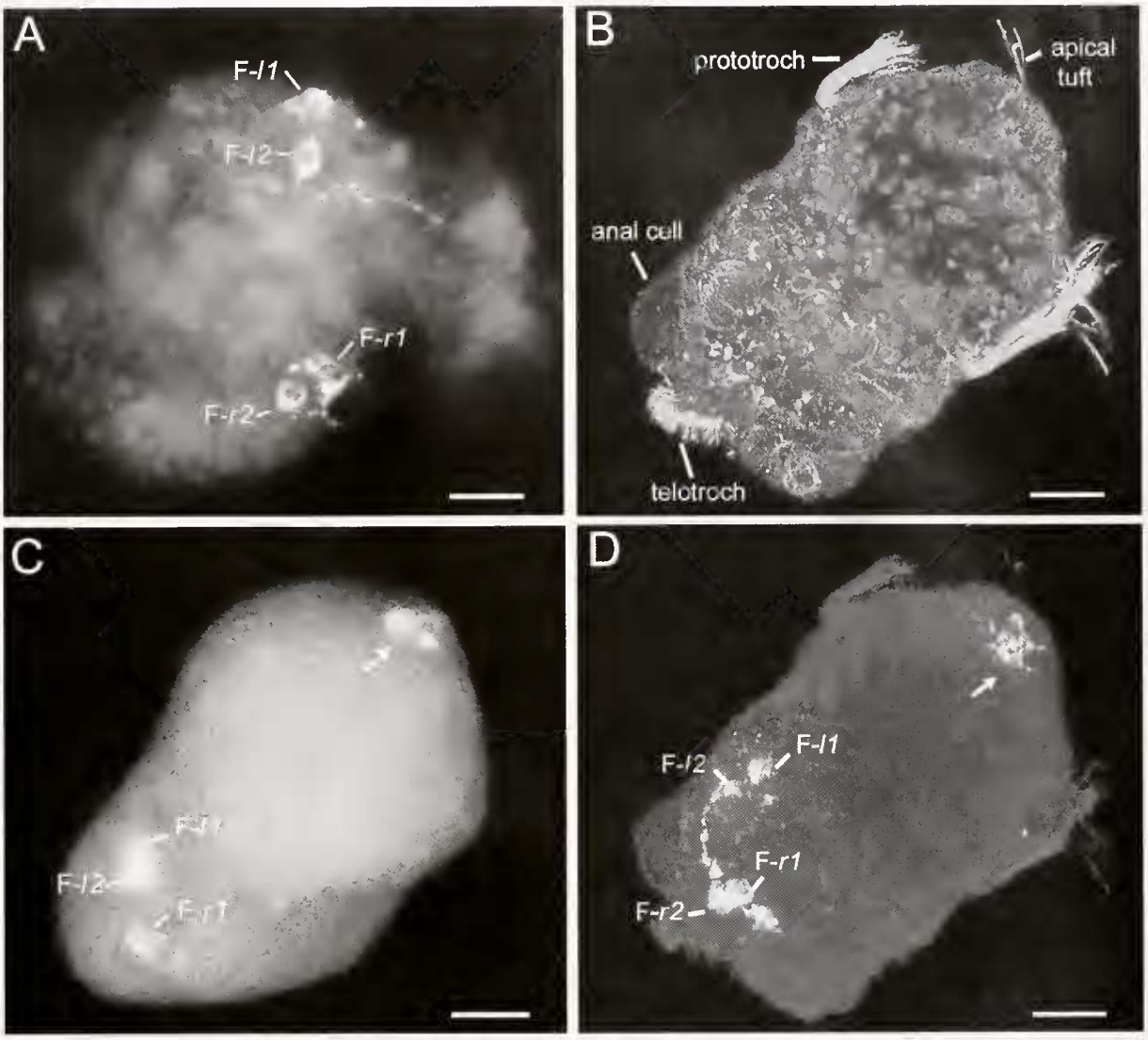

Figure 2. *Aplysia californica* during trochophore stages. Anterior is to the right in each figure. (A) Dorsal view of an embryo on day 2.5 showing the two pairs of posteriorly located FMRFamide-LIR cells with anterior projections. Scale bar = 20 μm. (B) Right lateral view of an embryo showing immunoreactivity for ∝-tubulin on day 3. Displays the locations of the apical tuft, prototroch, telotroch, and anal cell in the trochophore. Scale bar = 25 μm. (C) EFLRIamide-like immunoreactivity observed on day 3, showing F-*r*1, F-*l*1 and -*l*2 (which are not in focus), and the plexus under the apical tuft (arrow). Scale bar = 25 μm. (D) FMRFamide-like immunoreactivity observed in the same embryo as 2B showing F-*r*1, -*r*2, -*l*1 and -*l*2, and plexus under the apical tuft. Scale bar = 25 μm. C and D demonstrate the similar pattern of immunoreactivity for EFLRIamide and FMRFamide.

apical commissure toward the foot. Also by day 8, formaldehyde glutaraldehyde-induced fluorescence, indicative of catecholamine-containing cells, was observed in the foot region. The catecholamine-containing foot cells were vase-shaped and appeared in two bilaterally symmetric groups of two to three cells on each side (see day 9, Figs. 4B, 6A, B).

By day 9 additional FMRFamide-LIR cells were observed in positions consistent with the locations previously identified as the developing cerebral, pedal, and pleural ganglia of the future adult CNS in *A. californica* (Kriegstein, 1977; Marois and Carew, 1990; Marois and Carew, 1997b) and other opisthobranchs (Kempf *et al.*, 1997a). One of these FMRFamide central cells (FC-1) appeared near F-*r*1, another (FC-2) was located near F-*r*2, and a third (FC-3) was observed to the left of the apical commissure (Figs. 1D, 3C). Also by day 9, the number of catecholamine-containing cells increased to four to five cells on each side of the foot (Figs. 4B, 6A, 6B). Another pair of catecholamine-containing cells was located in the oral region. Catecholamine-containing fibers also extended from each

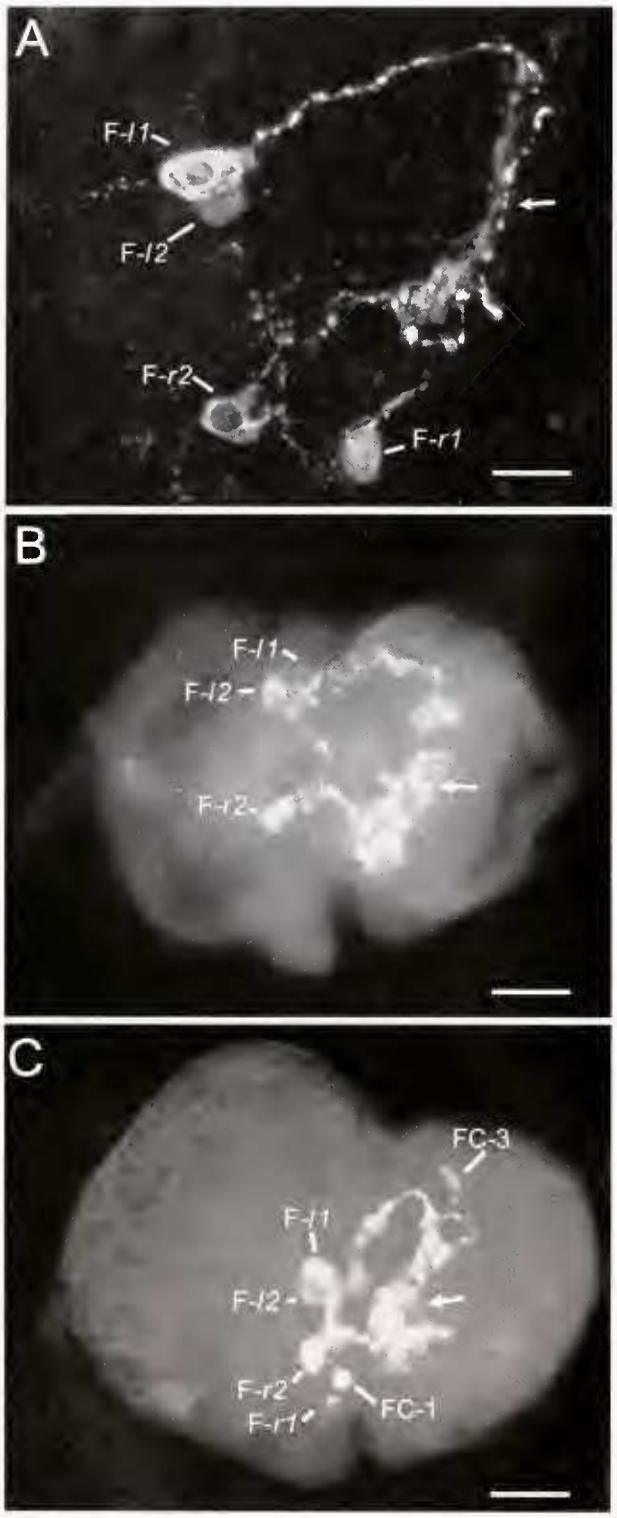

Figure 3. FMRFamide- and EFLRIamide-LIR cells and fibers in representative embryonic veliger stages of *Aplysia californica.* Anterior is to the right in each figure. Arrows indicate the FMRFamide-LIR plexus. (A) FMRFamide-like immunoreactivity observed on day 5, showing the asymmetry of F-*l*1, F-*l*2, F-*r*1, and F-*r*1. Scale bar = 15 μm. (B) Montage of two photographs showing the EFLRIamide-LIR cells and fibers on day 7. Scale bar = 28 μm. (C) FMRFamide-like immunoreactivity on day 9. Two additional cells, FC-1 and FC-3, are shown in this focus. Scale bar = 28 μm.

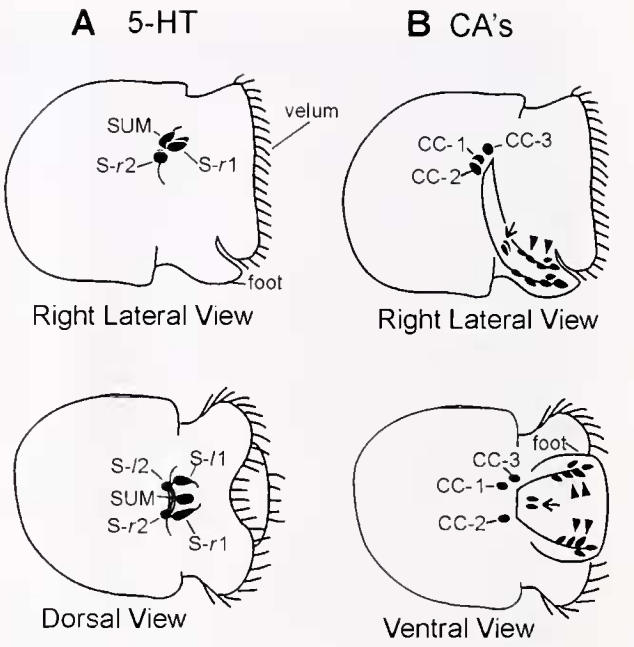

Figure 4. Schematic representations of serotonin-LIR and catecholamine-containing neurons in *Aplysia californica* during day 9. Top row: views from the right side and slightly superior to give a three-dimensional perspective. Anterior is to the right in each figure. (A) Serotonin-LIR cells (S-*l*1, S-*l*2, S-*r*1, S-*r*2, SUM) and fibers in the apical organ. Bottom row: dorsal view. (B) Cells and fibers containing catecholamines were located in the foot (arrow heads), oral region (arrow), and the region of the future CNS (CC-1, CC-2, CC-3). Bottom row: ventral view.

group of foot cells toward the region below the apical tuft where the FMRFamide-LIR apical commissure and serotonin-LIR plexus were located. In this region, three catecholamine-containing central cells were observed (Fig. 6B); two of these cells (CC-1, CC-2) were located on the right and another cell (CC-3) on the left. Unfortunately, the formaldehyde glutaraldehyde technique resulted in high background fluorescence, making it difficult to determine whether these cells were located in the apical organ or the developing cerebral or pedal ganglia of the future adult CNS. Also by this time, serotonin-LIR fibers could be seen projecting toward the velum, foot, and posterior region (Figs. 4A, 5A).

Discussion

The current study offers evidence that a relatively extensive nervous system forms during embryonic development of the opisthobranch gastropod *Aplysia californica.* The early nervous system includes posterior FMRFamide-LIR cells that first appear during the trochophore stage. By the veliger stage, serotonin-LIR cells appear in the apical organ (Marois and Carew, 1997a, b, c), and shortly before hatching catecholamine-containing cells appear around the mouth and in the foot. The first neurons within the developing

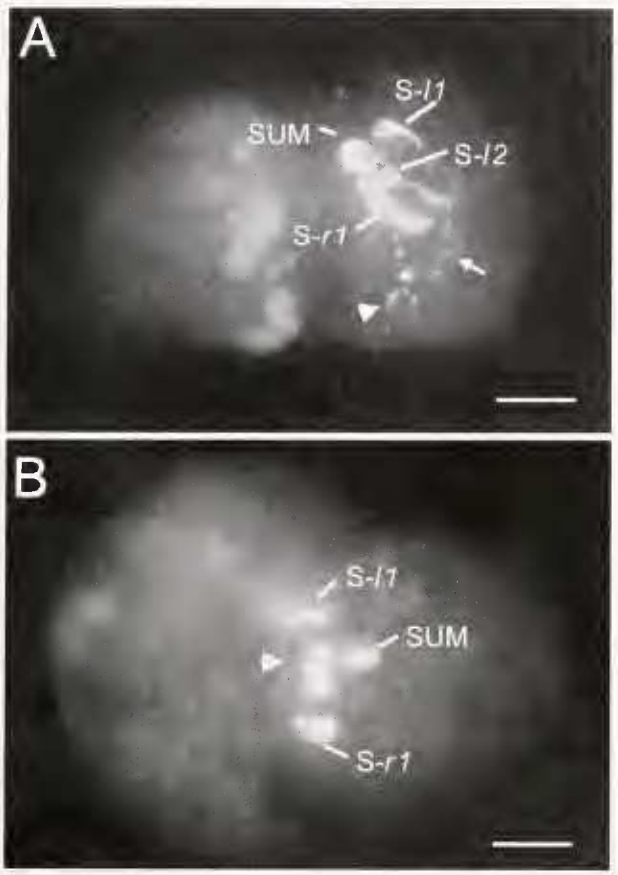

Figure 5. Serotonin-LIR cells and fibers in *Aplysia californica* during days 8–9. Anterior is to the right in each figure. (A) Right lateral view of an embryo on day 9 showing serotonin-LIR cells in the apical organ (S-*l*1, S-*l*2, S-*r*1, SUM) and fibers projecting into the foot (arrowhead) and velum (arrow). Scale bar = 30 μm. (B) Dorsal view of an embryo on day 8 showing three of the serotonin-LIR cells (S-*l*1, S-*r*1, SUM) of the apical organ. The apical commissure is indicated by the arrowhead. Scale bar = 28 μm.

ganglia, which will eventually constitute the adult CNS, only begin to appear during late embryonic stages (Schacher *et al.*, 1979). Such an arrangement of neuronal cells and fibers is similar to that found in representative pulmonate and prosobranch gastropod species (Croll and Voronezhskaya, 1996a; Dickinson *et al.*, 1999).

Posterior FMRFamide-LIR cells

Two pairs of FMRFamide-LIR cells appear in posterior regions and project anteriorly directed fibers in early trochophore stages of *A. californica*. FMRFamide-like immunoreactivity has also been observed posteriorly in early embryos of the other gastropod molluscs; however, differences were observed in the number and precise positions of these cells. *Lymnaea stagnalis* (Croll and Voronezhskaya, 1995, 1996a) and *Crepidula fornicata* (Dickinson *et al.*, 1999) each possess a single medial FMRFamide-LIR cell in posterior regions near the shell gland. No such cell was observed during embryonic development in *A. californica*. Nevertheless, it could have been missed if it was present only for a very brief period of development and if it did not exhibit reactivity to the anti-FMRFamide antibodies used in this study. During early veliger stages of *L. stagnalis*, additional FMRFamide-LIR posterior cells exist on the left and right fibers projecting from the most posterior medial cell (Croll and Voronezhskaya, 1995, 1996a). The location of these left and right cells in *L. stagnalis* may correspond to the left and right pairs of FMRFamide-LIR posterior cells in *A. californica*. FMRFamide-LIR cells in such lateral regions were not detected in *C. fornicata* (Dickinson *et al.*, 1999).

Despite variations in the number and position of posterior FMRFamide-LIR cells in the different species, they all share certain features. In all three species, the posterior

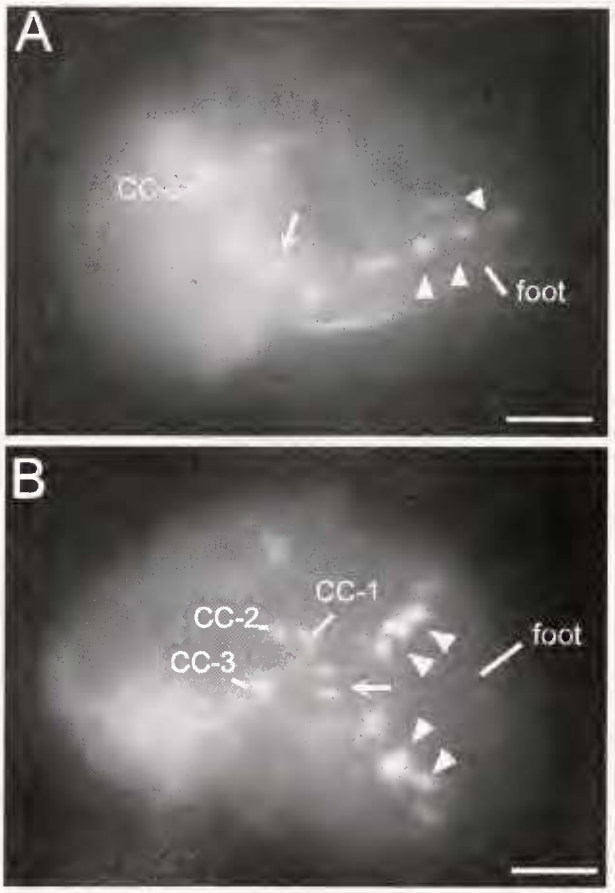

Figure 6. Catecholamine-containing cells and fibers in *Aplysia californica* during day 9. Anterior is to the right in each figure. (A) Right lateral view of an embryo showing catecholamine-containing cells in the foot (arrowheads), oral region (arrows), and apical region or region corresponding to the future ganglia of the adult CNS (CC-3). Scale bar = 28 μm. (B) Ventral view montage showing cells containing catecholamines. Cells are shown in the foot (arrowheads), oral region (arrows), and apical region or region corresponding to the future ganglia of the adult CNS (CC-1, CC-2, CC-3). Scale bar = 28 μm.

FMRFamide-LIR cells appear before any other nerve cells are detected. Also, the posterior FMRFamide-LIR cells all extend anteriorly directed axons that pass through the region in which the cerebral ganglia will later develop, and they eventually terminate in a region of the future pedal ganglia. FMRFamide-LIR fibers also appear under the apical tuft during trochophore stages of both *A. californica* and *C. fornicata*. This region later develops into the apical sensory organ, the underlying cerebral commissure, or both (Marois and Carew, 1997a, c). The plexus of immunoreactive fibers is extensive in this region, even at very early developmental stages. Although no other immunoreactive somata were detected at these stages, we cannot exclude the possibility that at least some fibers may derive from sources other than the posterior cells. In fact, FMRFamide-LIR fibers also branched repeatedly under the apical plate in early embryonic stages of *L. stagnalis*, but these fibers originate from nearby somata that exhibit little or no immunoreactivity (Croll and Voronezhskaya, 1996a).

Similarity also exists in the patterns of expression of the FMRFamide-related peptides in two species of molluscs. In both *L. stagnalis* and *A. californica*, the cells and fibers expressing FMRFamide- and EFLRIamide-like immunoreactivity are similar (Voronezhskaya and Elekes, 1997). The FMRFamide antiserum is immunoreactive to several FMRFamide-related peptides (Gaus *et al.*, 1993), whereas the EFLRIamide antiserum is immunoreactive only to EFLRIamide and FMRFamide itself (Santama *et al.*, 1995a, b, 1996). Our results thus indicate that immunoreactivity in early stages of *A. californica* is due to the presence of FMRFamide and EFLRIamide and not to the exclusive presence of other FMRFamide-related peptides. Antibodies against SEEPLY and the acidic peptide (ACP) that are processed from the FMRFamide precursor protein of *L. stagnalis* are not immunoreactive in embryonic *A. californica*. The sequence of SEEPLY is identical in *L. stagnalis* and *A. californica* (Greenberg and Price, 1992), but consistent with our findings in *A. californica*, Voronezhskaya and Elekes (1997) reported no detectable occurrence of this peptide in FMRFamide-LIR cells during embryonic stages of *L. stagnalis*. Conversely, ACP has not been isolated in *A. californica*. Therefore, the lack of positive immunoreaction for this latter peptide may be attributed to species-specificity of the amino acid sequence.

The aim of the present study was to examine the ontogeny of the nervous system during embryonic development, thus representing times much earlier than typically examined in *A. californica*. Since we did not examine later stages, the fates of the FMRFamide-LIR cells are unclear. It seems possible, however, that at least some of these FMRFamide-LIR cells and fibers later become incorporated in the ganglia and connectives of the CNS. The regions where the ganglia of the adult CNS are located have been previously identified in later veliger stages of *A. californica* (Kriegstein, 1977; Marois and Carew, 1990, 1997b). In the present study, FMRFamide-LIR cells can be observed in positions that correspond to these regions. For example, F-*l*1 and F-*l*2 seem to lie in positions that correlate to the future site of the left abdominal ganglia. F-*r*2 appears in the region where the osphradium will develop, whereas FC-2 seems to lie in a position that corresponds to the location of the right abdominal ganglia. F-*r*1, FC-1, and FC-3 appear in regions where the pleural or pedal ganglia will develop. Confirmation of the locations of these FMRFamide-LIR cells within the CNS must, however, await additional histological examination to identify the boundaries of the developing central ganglia. Although our work may have tentatively identified individual central neurons at earlier stages than previously demonstrated, the findings are still consistent with other descriptions of gangliogenesis. The presence of cerebral and pedal cells has been previously reported in hatchlings of *A. californica* (Kriegstein, 1977; Marois and Carew, 1997b) and other gastropods (D'Asaro, 1969; Page, 1992a, b; Lin and Leise, 1996b; Dickinson *et al.*, 1999). Anlagen of the visceral loop ganglia have also been identified in hatching stages of *A. californica* (Schacher *et al.*, 1979).

It is also possible that at least some posterior FMRFamide-LIR cells lie outside the developing ganglia and transiently express their transmitter phenotype during a short phase of embryogenesis. Such a fate for the early posterior FMRFamide-LIR cells has been shown in the developing embryos of *L. stagnalis* (Croll and Voronezhskaya, 1995; Voronezhskaya and Elekes, 1996).

Whatever the exact locations and fates of the various somata, FMRFamide-LIR fibers clearly mark pathways that span the length and breadth of the embryos in *A. californica*, and they may play a role in pioneering the various commissures and connectives of the adult nervous system. Such a role for early FMRFamide-LIR cells and fibers has been suggested in other molluscs such as *L. stagnalis* (Croll and Voronezhskaya, 1995, 1996a) and *C. fornicata* (Dickinson *et al.*, 1999) and is consistent with hypothesized roles of early developing fibers in other invertebrate groups such as insects (Bate, 1976; Caudy and Bentley, 1986; Goodman and Shatz, 1993) and annelids (Lacalli, 1981, 1982). The necessity of such pioneering fibers for the normal development of the CNS in gastropods must be tested in future experiments.

Serotonergic apical cells

The serotonin-LIR cells in the apical organ of *A. californica* have been previously described in detail (Croll and Voronezhskaya, 1995; Marois and Carew, 1997a, b, c). A similar arrangement of serotonin-LIR cells was also reported in other opisthobranchs (Kempf *et al.*, 1997a), prosobranchs (Dickinson *et al.*, 1999), and bivalves (Croll *et al.*, 1997).

The apical organ in molluscs and other invertebrate larvae may control larval behaviors such as swimming, feeding, and crawling (Leise, 1996; Lin and Leise, 1996a, b). Morphological evidence supports such hypothesized functions since serotonin-LIR cells of the apical organ innervate the velum in *A. californica* (Marois and Carew, 1997b, c) and other molluscs (Kulakovskiy and Flyachinskaya, 1994; Croll *et al.*, 1997; Kempf *et al.*, 1997; Dickinson *et al.*, 1999). Such velar innervation probably controls locomotion and feeding currents generated by cilia, which are responsive to serotonin in both adult forms (Audesirk *et al.*, 1979; Murakami, 1987; Syed and Winlow, 1989) and larval stages (Koshtoyants *et al.*, 1961; Beiras and Widdows, 1995). The apical organ is also thought to control the transduction of the metamorphic signal (Hirata and Hadfield, 1986; Couper and Leise, 1996; Hadfield *et al.*, 2000) and possibly to influence subsequent development of the adult CNS (Lacalli, 1981, 1994; Marois and Carew, 1997c).

Although non-serotonergic apical cells were not identified in the present study, other neurons in the apical organ of *A. californica* were previously identified using electron microscopy (Marois and Carew, 1997b). Flask-shaped, FMRFamide-LIR cells have also been observed in the apical region of the pulmonate *L. stagnalis* (Croll and Voronezhskaya, 1996a), the prosobranch *C. fornicata* (Dickinson *et al.*, 1999), and the opisthobranch *Phestilla sibogae* (Kempf *et al.*, 1992). It is possible that apical somata exhibit other transmitters or peptides in later stages of development in *A. californica.*

Anterior catecholaminergic cells

Catecholamine-containing cells exist in the foot region of *A. californica.* Similar cells also appeared in the foot of the gastropods *L. stagnalis* (Voronezhskaya *et al.*, 1999) and *C. fornicata* (Dickinson *et al.*, 1999), and in the bivalves *Mytilus edulis* and *Placopecten magellanicus* (Croll *et al.*, 1997). Such cells may be involved in metamorphosis, because catecholamines were found to modulate or induce metamorphosis in both gastropods (Pires *et al.*, 2000) and bivalves (Coon and Bonar, 1986). Similarly located cells have also been found to be responsive to the chemical cues inducing metamorposis in the opisthobranch *Onchidoris bilamellata* (Arkett *et al.*, 1989).

Catecholamine-containing cells are also found near the mouth of embryonic *A. californica,* consistent with similar cells reported in pulmonates, prosobranchs, and bivalves (Croll *et al.*, 1997; Dickinson *et al.*, 1999; Voronezhskaya *et al.*, 1999). The role for these cells is also unknown, although it is tempting to suggest that they might influence feeding behavior, which already displays a significant degree of sophistication within larval stages of molluscs (Baldwin and Newell, 1995).

We suggest that at least some other catecholamine-containing cells found in later larval stages might lie within the developing central ganglia, consistent with previous discussions of other central neurons in this paper. Confirmation of the locations of these cells must, however, await further experimentation to identify the boundaries of the developing central ganglia during embryonic stages.

Torsion and neurodevelopment

It was previously hypothesized that the opisthobranch nervous system lacks chiastoneury, or the twisting of the visceral loop into a figure-eight pattern, because neurodevelopment begins after torsion (Kandel *et al.*, 1981). However, the present study shows posterior neurons and fibers before the onset of torsion in *A. californica,* and if such neurons are later incorporated in the developing ganglia, a reevaluation of this hypothesis may be necessary. In early stages of the prosobranch *C. fornicata,* chiastoneury was observed in a figure-eight pattern of the FMRFamide-LIR fibers (Dickinson *et al.*, 1999). In corresponding stages of *A. californica,* such a figure-eight pattern did not exist, probably due to the different degrees of torsion in the two groups: 120-degree rotation in the opisthobranchs compared with the 180-degree rotation in the prosobranchs (Kandel, 1979; Kandel *et al.*, 1981). Cells and fibers may also be positioned more anteriorly and the connectives between ganglia may be shorter in opisthobranchs than in prosobranchs. Detorsion in opisthobranchs (Kandel, 1979; Kandel *et al.*, 1981) presumably eliminates much of the remaining evidence of twisting of the neural pathways.

Conclusion

The present study provides morphological evidence of neuronal elements that appear earlier in development than any previously identified neural structure, including the apical organ, in *A. californica.* Many catecholamine-containing cells are also identified in anterior regions of early embryonic stages. Together these elements (posterior cells, apical organ, and anterior catecholaminergic cells) may compose a distinct, primary larval nervous system that may be responsible for the control of several embryonic and larval behaviors such as swimming, feeding, and metamorphosis. The fate of such a primary larval nervous system is unknown, but evidence suggests that some neurons and connectives may be incorporated into the adult CNS while other cells disappear. Other invertebrate larvae from taxa such as polychaetes, nemertines, echinoderms, and phoronids have embryonic nervous systems that are very similar to those of molluscs (Hay-Schmidt, 1990a, b, c; 1995).

This report also suggests the possibility that the larval nervous system may form a scaffold along which the adult nervous system develops. Such a hypothesis is consistent with the suggested role of FMRFamide-LIR cells and processes in corresponding stages of other gastropods and other

invertebrate groups. The demonstration of early developing neurons and their transmitter phenotypes in *A. californica* opens new opportunities for a deeper understanding of the ontogeny and phylogeny of both behavior and neuronal function in this important model species.

Acknowledgments

We thank J. Nason for technical assistance and L. Nezlin for critical readings of this manuscript. Funding was provided by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) to R.P.C. (grant number: OGP0038863) and from the Russian Fund for Basic Research (RFBR) to E.E.V. (grant number 99-04-48415).

Literature Cited

Arkett, S. A., F.-S. Chia, J. I. Goldberg, and R. Koss. 1989. Identified settlement receptor cells in a nudibranch veliger respond to specific cue. *Biol. Bull.* **176:** 155–160.

Audesirk, G., R. E. McCaman, and A. O. D. Willows. 1979. The role of serotonin in the control of pedal ciliary activity by identified neurons in *Tritonia diomedea*. *Comp. Biochem. Physiol.* **62:** 87–91.

Baldwin, N. S., and R. I. E. Newell. 1995. Feeding rate responses of oyster larvae (*Crassostrea virginica*) to seston quantity and composition. *J. Exp. Mar. Biol. Ecol.* **189:** 77–91.

Bate, C. M. 1976. Pioneer neurons in an insect embryo. *Nature* **260:** 54–55.

Beiras, R., and J. Widdows. 1995. Effects of the neurotransmitters dopamine, serotonin and norepinephrine on the ciliary activity of mussel (*Mytilis edulis*) larvae. *Mar. Biol.* **122:** 597–603.

Bonar, D. B. 1978. Ultrastructure of a cephalic sensory organ in larva of the gastropod *Phestilla sibogae* (Aeolidacea, Nudibranchia). *Tissue Cell* **10:** 153–165.

Caudy, M., and D. Bentley. 1986. Pioneer growth cone steering along a series of neuronal and non-neuronal cues of different affinities. *J. Neurosci.* **6:** 1781–1795.

Coon, S. L., and D. B. Bonar. 1986. Norepinephrine and dopamine content of larvae and spat of the Pacific oyster, *Crassostrea gigas*. *Biol. Bull.* **171:** 632–639.

Couper, J. M., and E. M. Leise. 1996. Serotonin injections induce metamorphosis in larvae of the gastropod mollusc *Ilyanassa obsoleta*. *Biol. Bull.* **191:** 178–186.

Croll, R. P., and E. E. Voronezhskaya. 1995. Early FMRFamide-like immunoreactive cells in gastropod neurogenesis. *Acta Biol. Hung.* **46:** 295–303.

Croll, R. P., and E. E. Voronezhskaya. 1996a. Early elements in gastropod neurogenesis. *Dev. Biol.* **173:** 344–347.

Croll, R. P., and E. E. Voronezhskaya. 1996b. Early neurodevelopment in *Aplysia*, *Lymnaea* and *Helisoma*. *Soc. Neurosci. Abstr.* **22:** 1948.

Croll, R. P., D. L. Jackson, and E. E. Voronezhskaya. 1997. Catecholamine-containing cells in larval and postlarval bivalve molluscs. *Biol. Bull.* **193:** 116–124.

Croll, R. P., E. E. Voronezhskaya, L. Hiripi, and K. Elekes. 1999. Development of catecholaminergic neurons in the pond snail, *Lymnaea stagnalis* II: Postembryonic development of central and peripheral cells. *J. Comp. Neurol.* **404:** 285–296.

D'Asaro, C. N. 1969. The comparative embryogenesis and early organogenesis of *Bursa corrugata* Perry and *Distorsio clathrata* (Gastropoda: Prosobranchia). *Malacologia* **9:** 349–389.

Dickinson, A. J. G., J. Nason, and R. P. Croll. 1999. Histochemical localization of FMRFamide, serotonin and catecholamine in embryonic *Crepidula fornicata* (Prosobranchia: Gastropoda). *Zoomorphology* **119:** 49–62.

Furness, J. B., M. Costa, and A. J. Wilson. 1977. Water-stable fluorophores, produced by reaction with aldehyde solutions, for the histochemical localization of catechol- and indolethylamines. *Histochemistry* **52:** 159–170.

Gaus, G., K. E. Dohle, D. A. Price, M. J. Greenberg, T. D. Lee, and B.-A. Battle. 1993. The sequences of five neuropeptides isolated from *Limulus* using antisera to FMRFamide. *Biol. Bull.* **184:** 322–329.

Goodman, C. S., and C. J. Shatz. 1993. Developmental mechanisms that generate precise patterns of neuronal connectivity. *Cell* **72:** 77–98.

Greenberg, M. J., and D. A. Price. 1992. Relationships among the FMRFamide-like peptides. *Prog. Brain Res.* **92:** 25–37.

Hadfield, M. G., E. A. Meleshkevitch, and D. Y. Boudko. 2000. The apical sensory organ of a gastropod veliger is a receptor for settlement cues. *Biol. Bull.* **198:** 67–76.

Hay-Schmidt, A. 1990a. Distribution of catecholamine-containing, serotonin-like and neuropeptide FMRFamide-like immunoreactive cells and processes in the nervous system of the pilidium larva (Nemertini). *Zoomorphology* **109:** 231–244.

Hay-Schmidt, A. 1990b. Distribution of catecholamine-containing, serotonin-like and neuropeptide FMRFamide-like immunoreactive neurons and processes in the nervous system of the actinotroch larva of *Phoronis muelleri* (Phoronida). *Cell Tissue Res.* **259:** 105–118.

Hay-Schmidt, A. 1990c. Distribution of catecholamine-containing, serotonin-like and neuropeptide FMRFamide-like immunoreactive neurons and processes in the nervous system of the early actinotroch larva of *Phoronis vancouverensis* (Phoronida): distribution and development. *Can. J. Zool.* **68:** 1525–1536.

Hay-Schmidt, A. 1995. The larval nervous system of *Polygodius lacteus* Schlender, 1868 (Polygordiidae, Polychaeta): Immunocytochemical data. *Acta Zool. (Stockholm)* **76:** 121–140.

Hirata, K. Y., and M. G. Hadfield. 1986. The role of choline in metamorphic induction of *Phestilla* (Gastropoda, Nudibranchia). *Comp. Biochem. Physiol.* **84:** 15–21.

Jackson, A. R., T. W. MacRae, and R. P. Croll. 1995. Unusual distribution of tubulin isoforms in the snail *Lymnaea stagnalis*. *Cell Tissue Res.* **281:** 507–516.

Jacob, M. H. 1984. Neurogenesis in *Aplysia californica* resembles nervous system formation in vertebrates. *J. Neurosci.* **4:** 1225–1239.

Kandel, E. R. 1979. *Behavioral Biology of* Aplysia. W. H. Freeman, San Francisco.

Kandel, E. R., A. Kriegstein, and S. Schacher. 1981. Development of the central nervous system of *Aplysia* in terms of the differentiation of its specific identifiable cells. *Neuroscience* **5:** 2033–2063.

Kempf, S. C., G. V. Chun, and M. G. Hadfield. 1992. An immunocytochemical search for potential neurotransmitters in larvae of *Phestilla sibogae* (Gastropoda, Opisthobranchia). *Comp. Biochem. Physiol.* **101C:** 299–305.

Kempf, S. C., L. R. Page, and A. Pires. 1997. Development of serotonin-like immunoreactivity in the embryos and larvae of nudibranch mollusks with emphasis on the structure and possible function of the apical sensory organ. *J. Comp. Neurol.* **386:** 507–528.

Koshtoyants, K. S., G. A. Buznikov, and B. N. Manukhin. 1961. The possible role of 5-hydroxy-tryptamine in the motor activity of embryos of some marine gastropods. *Comp. Biochem. Physiol.* **3:** 20–26.

Kriegstein, A. R. 1977. Development of the nervous system of *Aplysia californica*. *Proc. Natl. Acad. Sci. USA* **74:** 375–378.

Kulakovskiy, E. Y., and L. P. Flyachinskaya. 1994. Formation of elements of the regulatory systems in larval development of *Mytilus edulis*. *Hydrobiol. J.* **30:** 85–94.

Lacalli, T. C. 1981. Structure and development of the apical organ in

trochophores of *Spirobranchus polycerus, Phyllodoce maculata* and *Phyllodoce mucosa* (Polychaeta). *Proc. R. Soc. Lond. B* **212:** 381–402.

Lacalli, T. C. 1982. The nervous system and ciliary band of Muller's larva. *Proc. R. Soc. Lond. B* **217:** 37–58.

Lacalli, T. C. 1994. Apical organs, epithelial domains and origin of the chordate central nervous system. *Am. Zool.* **34:** 533–541.

Leise, E. M. 1996. Selective retention of the fluorescent dye DASPEI in a larval gastropod mollusc after paraformaldehyde fixation. *Micro. Res. Tech.* **33:** 496–500.

Lin, M., and E. M. Leise. 1996a. NADPH-Diaphorase activity changes during gangliogenesis and metamorphosis in the gastropod mollusc *Ilyanassa obsoleta. J. Comp. Neurol.* **374:** 194–203.

Lin, M.-F., and E. M. Leise. 1996b. Gangliogenesis in the prosobranch gastropod *Ilyanassa obsoleta. J. Comp. Neurol.* **374:** 180–193.

Marois, R., and T. J. Carew. 1990. The gastropod nervous system in metamorphosis. *J. Neurobiol.* **7:** 1053–1071.

Marois, R., and T. J. Carew. 1997a. Fine structure of the apical ganglion and its serotonergic cells in the larva of *Aplysia californica. Biol. Bull.* **192:** 388–398.

Marois, R., and T. J. Carew. 1997b. Ontogeny of serotonergic neurons in *Aplysia californica. J. Comp. Neurol.* **386:** 477–490.

Marois, R., and T. J. Carew. 1997c. Projection patterns and target tissues of serotonergic cells in larval *Aplysia californica. J. Comp. Neurol.* **386:** 491–506.

Marois, R., and R. P. Croll. 1992. Development of serotonergic cells within the embryonic central nervous system of the pond snail, *Lymnaea stagnalis. J. Comp. Neurol.* **322:** 255–265.

Mescheryakov, V. N. 1990. The common pond snail *Lymnaea stagnalis* L. Pp. 69–132 in *Animal Species for Developmental Studies,* D. A. Dettlaff and S. G. Vassetzky, eds. Plenum Press, New York.

Murakami, A. 1987. Control of ciliary beat frequency in the gill of *Mytilus. Comp. Biochem. Physiol.* **86C:** 273–279.

Page, L. R. 1992a. New interpretation of a nudibranch central nervous system based on ultrastructural analysis of neurodevelopment in *Melibe leonina.* I. Cerebral and visceral loop ganglia. *Biol. Bull.* **182:** 348–365.

Page, L. R. 1992b. New interpretation of a nudibranch central nervous system based on ultrastructural analysis of neurodevelopment in *Melibe leonina.* II. Pedal, pleural, and labial ganglia. *Biol. Bull.* **182:** 366–381.

Page, L. R., and S. C. Parries. 2000. Comparative study of the apical ganglion in planktotrophic caenogastropod larvae: ultrastructure and immunoreactivity to serotonin. *J. Comp. Neurol.* **418:** 383–401.

Pires, A., R. P. Croll, and M. G. Hadfield. 2000. Catecholamines modulate metamorphosis in the opisthobranch gastropod, *Phestilla sibogae. Biol. Bull.* **198:** 319–331.

Raineri, M. 1995. Is a mollusc an evolved bent metatrochophore? A histochemical investigation of neurogenesis in *Mytilus* (Mollusca:Bivalvia). *J. Mar. Biol. Assoc. U.K.* **75:** 571–592.

Raineri, M., and M. Ospovat. 1994. The initial development of gangliar rudiments in a posterior position in *Mytilus galloprovincialis* (Mollusca: Bivalvia). *J. Mar. Biol. Assoc. U.K.* **74:** 73–77.

Santama, N., K. W. Li, K. E. Bright, M. Yeoman, W. P. Geraerts, P. R. Benjamin, and J. F. Burke. 1993. Processing of the FMRFamide precursor protein in the snail *Lymnaea stagnalis:* characterization and neuronal localization of a novel peptide, "SEEPLY." *Eur. J. Neurosci.* **5:** 1003–1016.

Santama, N., P. R. Benjamin, and J. F. Burke. 1995a. Alternative RNA splicing generates diversity of neuropeptide expression in the brain of the snail *Lymnaea: in situ* analysis of mutually exclusive transcripts of the FMRFamide gene. *Eur. J. Neurosci.* **7:** 65–76.

Santama, N., C. H. Wheeler, D. R. Skingsley, M. S. Yeoman, K. Bright, I. Kaye, J. F. Burke, and P. R. Benjamin. 1995b. Identification, distribution and physiological activity of three novel neuropeptides of *Lymnaea:* EFLRIamide and pQFYRIamide encoded by the FMRFamide gene, and a related peptide. *Eur. J. Neurosci.* **7:** 234–246.

Santama, N., K. W. Li, W. P. Geraerts, P. R. Benjamin, and J. F. Burke. 1996. Post-translational processing of the alternative neuropeptide precursor encoded by the FMRFamide gene in the pulmonate snail, *Lymnaea stagnalis. Eur. J. Neurosci.* **8:** 968–977.

Schacher, S., E. R. Kandel, and R. Woolley. 1979. Development of neurons in the abdominal ganglion of *Aplysia californica* I. Axosomatic synaptic contacts. *Dev. Biol.* **71:** 176–190.

Smith, S. A., J. Nason, and R. P. Croll. 1998. Distribution of catecholamines in the sea scallop, *Placopecten magellanicus. Can. J. Zool.* **76:** 1254–1262.

Syed, N. I., and W. Winlow. 1989. Morphology and electrophysiology of neurons innervating the ciliated locomotor epithelium in *Lymnaea stagnalis* (L.). *Comp. Biochem. Physiol.* **93A:** 633–644.

Voronezhskaya, E. E., and K. Elekes. 1996. Transient and sustained expression of FMRFamide-like immunoreactivity in the developing nervous system of *Lymnaea stagnalis. Cell Mol. Neurobiol.* **16:** 661–676.

Voronezhskaya, E. E., and K. Elekes. 1997. Expression of FMRFamide gene neuropeptides is partly different in the embryonic nervous system of the pond snail, *Lymnaea stagnalis* L. *Neurobiology* **5:** 91–93.

Voronezhskaya, E. E., L. Hiripi, K. Elekes, and R. P. Croll. 1999. Development of catecholaminergic neurons in the pond snail, *Lymnaea stagnalis* I: Embryonic development of dopaminergic neurons and dopamine-dependent behaviors. *J. Comp. Neurol.* **404:** 297–309.